TECHNICAL PROGRESS REPORT

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Project Title: Large Scale Solubilization of Coal and Bioconversion to Utilizable Energy

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Introduction

In order to develop a system for a large scale coal solubilization and its bioconversion to utilizable fuel, we plan to clone the genes encoding *Neurospora* protein that facilitate depolymerization of coal. We also plan to use desulfurizing bacteria to remove the sulfur *in situ* and use other microorganisms to convert biosolubilized coal into utilizable energy following an approach utilizing several microorganisms (Faison, 1991). In addition the product of coal solubilized by fungus will be characterized to determine their chemical nature and the mechanism of reaction catalyzed by fungal product during *in vivo* and *in vitro* solubilization by the fungus or purified fungal protein.

Main Objectives

1. Cloning of *Neurospora* gene for coal depolymerization protein controlling solubilization in different host cells, utilizing *Neurospora* plasmid and other vector(s).

2. a. Development of a large scale electrophoretic separation of coal derived products obtained after microbial solubilization.

   b. Identification of the coal derived products obtained after biosolubilization by *Neurospora* cultures or obtained after *Neurospora* enzyme catalyzed reaction in *in vitro* by the wildtype and mutant enzymes.


4. Characterization of *Neurospora* wildtype and mutant CSA protein(s) involved in solubilization of coal in order to assess the nature of the mechanism of solubilization and the role of *Neurospora* proteins in this process.
Methods:

Only experimental approaches for objective #1 are presented here since experiments were performed during this period in this area.

Objective #1   Cloning of gene for Neurospora CSA-protein

The following methods will be used to clone the gene for Neurospora protein with coal solubilization activity (CSA).

A. Identification of Neurospora CSA protein gene in a DNA library:

In case the above approach to clone Neurospora gene in yeast or Neurospora cells is not successful, alternative methods will be used. The wildtype CSA protein has been purified in my laboratory. The purified wild type CSA protein obtained from the SDS PAGE (Laemmli, 1970) will be electroblotted onto Immobilon P, polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA) using the method of Matsudaira (1987). The membrane will then be given to Dr. Ishikawa of the Protein Microanalyses Facility of the Carolina Institute for Biological Research and Technology (IBRT), University of South Carolina, Columbia campus for microsequencing. Based on about the first nine amino acid sequences at the N-terminus (Matsudaira 1987) or internal amino acid sequence (Huang, 1983), an oligonucleotide probe will be synthesized at the oligonucleotide synthesis facility of the USC IBRT facility located in our Department. This oligonucleotide probe will then be used to screen a cDNA library (as well as a genomic library of Neurospora) to identify the clone carrying Neurospora CSA protein gene.

Alternatively, immunoblotting will be used to identify clone with Neurospora CSA gene. We will prepare polyclonal antibody against the Neurospora CSA protein in rabbit. The antibody so prepared will be used to screen a Neurospora cDNA library to
identify a clone carrying the gene for the Neurospora CSA protein. This method should work since the genes in the cDNA library are known to be expressed. Once the clone containing the gene for the Neurospora CSA protein is identified, the gene will be transferred to suitable vector such as pYELeu-10 or to Neurospora psp 2.2, a mt DNA plasmid to which benomyl resistance (Ben\textsuperscript{n}) gene has been added as a selectable marker (our unpublished results).

These chimeric plasmid containing the Neurospora CSA protein gene will be used to transform yeast cells or Neurospora (sol) mutant cells (see Figure 2), which are devoid of ability to biosolubilize coal in vivo. The transformants will be identified by their ability to solubilize coal in vivo when assayed in Petri plates. The yeast or Neurospora transformants will be further examined for multiple copy of the CSA protein gene and for their possible autonomous existence by the method of Southern hybridization or by amount of the CSA protein produced.

The plasmids pYELeu-2 and psp 2.2 and the genomic and cDNA libraries of Neurospora and PCR machine are available in my laboratory. All methods of molecular cloning, transformation, and identification and Characterization of transformants will be as practiced in my laboratory (Schablik et al., 1982; Almasan and Mishra, 1988; 1990; 1991) or as described previously (Hinnen et al., 1978; Maniatis et al., 1989; Yadav and Mishra, 1994; Feher and Mishra, 1994).

B. Cloning of Neurospora gene in yeast

As yeast cannot solubilize coal this provides the easiest way to clone Neurospora gene by shotgun experiment in which Neurospora DNA segment (obtained after restriction enzyme digestion) will be ligated to pYELeu-2 plasmid and
then introduced into CaCl₂ treated competent pYELeu-2 yeast cells (Hinnen et al., 1978). The Leu⁺ yeast transformants will be examined for their acquisition of ability to solubilize coal in plate assays. The transformed yeast colonies containing the chimeric plasmid carrying Neurospora DNA segment encoding CSA-protein will be thus identified and further characterized. The success of this shotgun-transformation experiment using yeast recipient cell will depend on the expression of the Neurospora gene encoding CSA protein in yeast cells. Since a number of heterologous genes have been expressed in yeast, it is therefore expected that Neurospora CSA protein gene could be expressed in yeast (Mishra, 1985; 1991).

The wildtype Neurospora gene for CSA will also be directly cloned in Neurospora mutant cells deficient for CSA activity. In this approach wildtype Neurospora DNA will be shotgunned into a Neurospora plasmid pst 2.2 Ben⁸ (Ben⁸ confers resistance to antibiotic benomyl); the chimeric plasmid will be used to transform Neurospora mutant lacking CSA (see Figure 2). First the Ben⁸ transformants will be picked up by their ability to grow on plates containing benomyl and then these will be examined for coal solubilization activity (CSA).
**Results:**

Following experiments have been performed to achieve the proposed goals:

A. **Further Purification of Neurospora CSA protein.** A purification of the Neurospora protein with coal solubilization activity (CSA) was undertaken in order to determine its N-terminal or internal amino acid sequence or to prepare the antibody against this protein in rabbit. The amino acid sequence will be used to prepare the oligonucleotides to identify the clone carrying Neurospora CSA gene among cDNA organomic libraries. Alternatively, the antibody will be used to identify the clone carrying Neurospora CSA gene via immunoblotting. Even though this protein was purified in microscale in my laboratory by a previous graduate student (Brian Odom) who worked on this aspect, he has left, after receiving his Ph.D. to join as Assistant Professor at Georgia Southern University. I have now been joined by a new graduate student, Ashish Patel, who has no knowledge in working with microorganisms. I have therefore attempted this purification of CSA protein in order to give Mr. Patel first hand knowledge of enzyme purification so that he could eventually use it for determining the amino acid sequence of this protein and for preparation of oligonucleotide probe and for raising antibody against this protein needed for use by him during the cloning of CSA gene.

We have finally devised a methodology to purify the Neurospora CSA protein on a large scale. The purification protocol includes fractionation of a large volume (3-10 litre) of culture filtrate in which Neurospora has been grown by DEAE cellulose chromatography, Biogel chromatography, and phenyl
Sepharose chromatography. At each step of chromatography, the peak fractions with high coal solubilization activity (CSA) was pooled and then utilized for subsequent chromatographic procedures. The coal solubilization is monitored via an increase in absorption at 254 nm due to release of UV absorbing material from coal added to the reaction mixture containing Neurospora protein. We are prompted to try this new approach to purify CSA protein because of our recent success in purifying another difficult protein (Feher and Mishra, 1994). Using this new protocol for protein purification one homogenous peak of protein with CSA property has been obtained. This Neurospora preparation was found to possess only one protein when analyzed on SDS polyacrylamide gel. We have confirmed the solubilization process of the coal by Neurospora crassa and have developed a protocol for the purification of the enzyme (see Table 1) which shows intense solubilization of coal (see Figure 1). He has also been successful in purifying the enzyme in large scale after ammonium sulfate precipitation. We have been joined by Dr. Y.P. Chen in the purification of protein to homogeneity. Dr. Chen is an Associate Research Professor and Director of Fermentation and Protein Purification facility in our department. His collaboration has already proven to be useful. We are now attempting to obtain a large amount of Neurospora CSA protein (i.e., in mg amount) for the preparation of antibody in the rabbit against this protein. We are also planning to get the amino terminal sequence of this protein in order to design the oligonucleotides for the primer to be used in the experiments for the cloning of the gene for CSA protein.
B. **Determination of the nature of the enzymatic activity of the *Neurospora* CSA protein.** We have shown that the purified *Neurospora* CSA protein is a phenol-oxidase. This has been demonstrated by the ability of *Neurospora* proteins to oxidize tyrosine or cathachol (in presence of ascorbic acid). Such demonstration of the nature of enzymatic reaction catalyzed by the *Neurospora* enzyme is very important. The results of these experiments clearly establish the enzymatic role of the enzyme and rules out the possibility that *Neurospora* protein may solubilize the coal by acting as a metal chelator. The fact that *Neurospora* protein is a tyrosinase (and phenol oxidase) was further established by the demonstration that commercially available tyrosinase (obtained from Sigma Chemical) can solubilize the coal in our laboratory. Now we are planning to establish the identity of *Neurospora* CSA protein with tyrosinase by immunological methods using antibody against Tyrosinase to block the CSA property of *Neurospora* protein.

The assay conditions for determination of tyrosinase and phenol oxidase were as presented in Appendix 1. The coal solubilization activity of commercial tyrosinase was determined as carried out in our laboratory.

C. **Development of transformation system.** Mr. Patel has also continued the project to make vector DNA, genomic DNA, and to prepare Sphaeroplasts of *Neurospora* mycelia so that he can use them for the molecular cloning experiments. The sphaeroplasts were made using cell wall digesting enzyme (Novozyme) in isotonic buffer to avoid the lysis of the cells. He is trying several conditions to optimize the frequency of molecular cloning.
D. **Preparation of the vector DNA.** We have described a plasmid (pstpb 2.2) (to which a selectable marker gene for resistance to benomyl has been added). We have prepared this vector plasmid for the molecular cloning experiments. We have developed methodology to prepare this plasmid in microgram amounts. Mr. Patel plans to shotgun *Neurospora* genomic DNA into this vector plasmid and then transfer the mutant strain fo *Neurospora* lacking CSA) into colonies capable of CSA (i.e., coal solubilization activity).

E. **Analysis of the product of coal solubilization.** Coal samples were solubilized biologically by *Neurospora crassa* as well as chemically by hydrogen peroxide and by NaOH. The products after solubilization were separated by gel electrophoresis and then compared. The products of the alkali solubilized coal in processing a high mobility band on agarose gel that can be visualized in UV light; the H$_2$O$_2$ solubilized coal lacked this UV fluorescent band. These experiments indicate that biological solubilization is similar to alkali solubilization in retaining an aromatic rich component latter is however destroyed by H$_2$O$_2$ treatment. In addition, Mr. Patel has developed a methodology to isolate the product of coal solubilization on a large scale which would now provide us with enough samples for chemical analysis and NMR studies. He has also examined the solubility of the coal derived products in a large number of organic solvent; of these alcohol is most useful. The product of coal solubilization has been identified by mass spectroscopy and demonstrated to be low molecular substance (see Table 2). The nature of the coal product obtained after biosolubilization clearly provides evidence for the enzymatic
activity of the *Neurospora* protein. We have further demonstrated that coal solubilized by commercial tyrosinase also yield products similar to that produced by the *Neurospora* CSA protein.

F. **Personnel.** The work is carried by Mr. A. Patel and Dr. Mishra (PI). Dr. Y.P. Chen has also joined the project as a collaborator. The data reported here was presented at the annual meetings of the DOE contractors in Nashville and at the meetings in Chicago this summer of the Chemical Society of America for which the PI was invited.

9. **Literature References**


**Fig. 1.** *In vitro* solubilization of coal by purified *Neurospora* mark the intense colorogenic substance released due to biosolubilization of coal (left picture) in the presence of *Neurospora* proteins as compared to the blank (right picture) when the reaction mixture did not contain *Neurospora* protein preparation.
Table 1.

PURIFICATION STEPS FOR NEUROSPORA PROTEIN RESPONSIBLE FOR COAL SOLUBILIZATION

LIQUID CULTURE GROWN IN VOGEL’S MINIMAL MEDIA. SEPARATED THE MYCELIIUM.

↓

PRECIPITATION OF FILTRATE (CRUDE EXTRACT) WITH 90% AMMONIUM SULFATE.

↓

CENTRIFUGATION AT 6000 RPM FOR 20 MINUTES TO SEPARATE THE PROTEIN (PELLET).

↓

ADDITION OF BISTRIS (50mM, pH-7.0) TO THE PELLET.

↓

TREATMENT WITH COAL TO CHECK ACTIVITY. INCREASE IN ACTIVITY IS CORRELATED TO INCREASE IN ABSORBANCE AT 254 nm.

↓

TREATMENT WITH SEPHADEX G-25 AND SUBSEQUENT DIALYSIS TO REMOVE ALL IMPURITIES.

↓

REPETITION OF ACTIVITY ASSAY FOR CONFIRMATION OF SOLUBILIZATION PROCESS.

↓

LOADING OF ENZYME EXTRACT ON DE-52 (DEAE-CELLULOSE) 3.8 cm X 11 cm COLUMN PRE-EQUILIBRATED WITH PHOSPHATE BUFFER.

↓

ELUTION OF PROTEINS WITH A LINEAR GRADIENT OF SODIUM CHLORIDE FROM 0.0-1M IN PHOSPHATE BUFFER.

↓

CONCENTRATION OF ENZYME PEAK BY ULTRAFILTRATION.

↓

APPLICATION TO BIO-GEL (PHENYLSEPHAROSE) 1.8 X 68 cm COLUMN PRE-EQUILIBRATED WITH PHOSPHATE BUFFER.

↓

ANALYSIS BY SDS-GEL ELECTROPHORESIS.
### CHARACTERISTICS OF COAL DERIVED PRODUCTS

<table>
<thead>
<tr>
<th>TYPE OF PRODUCT</th>
<th>MOLECULAR WEIGHT</th>
<th>POSSIBLE FORMULAS</th>
<th>INFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROTEIN FROM LIQUID CULTURE &amp; COAL</td>
<td>84</td>
<td>C₆H₁₂</td>
<td>May be a contaminant like sugar molecules present on growth medium</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>C₆H₁₀</td>
<td>Hydrocarbons</td>
</tr>
<tr>
<td></td>
<td>326</td>
<td>C₂₃H₄₆, C₂₄H₃₈, or C₂₆H₁₄</td>
<td>Hydrocarbons</td>
</tr>
<tr>
<td></td>
<td>278</td>
<td>C₁₆H₂₂O₄</td>
<td>May be a contaminant (Phthalate) from the plastic tubes used during the handling of samples.</td>
</tr>
<tr>
<td>IN VIVO SOLUBILIZED COAL</td>
<td>123</td>
<td>CH₂CH₂OH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>164</td>
<td>C₁₁H₁₆O</td>
<td></td>
</tr>
<tr>
<td></td>
<td>216</td>
<td>C₁₂H₂₄O₃</td>
<td></td>
</tr>
<tr>
<td>IN VIVO SOLUBILIZED COAL RAN ON AGAROSE GEL TO SEPARATE THE INORGANIC AND ORGANIC (FLUORESCENT) PARTS. ANALYSIS OF THE ORGANIC PART.</td>
<td>216</td>
<td>C₁₂H₂₄O₃</td>
<td></td>
</tr>
<tr>
<td></td>
<td>230</td>
<td>O - O \ R-O-C-(CH₂)₄-C-OR</td>
<td>Hexanedioic Acid, Bis(1-Methyl Ethyl) Ester</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\ or \ CH₃</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>\ R=CH \ CH₃</td>
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<td></td>
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<td>\ or \</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>\ R=CH₂CH₂CH₃</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX

Methods for the assay of phenol oxidase and tyrosinase activities in *Neurospora* CSA protein preparation.

Know tyrosinase (phenol oxidase) obtained from Sigma Co. St. Louis was used as control.
SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of TYROSINASE
Polyphenol Oxidase Activity
(EC 1.14.18.1)

PRINCIPLE:
L-DOPA + O₂ + Ascorbic Acid → Tyr. 6-MeDopaquinone + H₂O + Dehydro-Ascorbic Acid

Abbreviations used:
TYR = Tyrosinase
L-DOPA = L-3,4-Dihydroxyphenylalanine

CONDITIONS: T = 25°C, pH 6.5, A₂₆₅nm, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 50 mM Potassium Phosphate Buffer, pH 6.5 at 25°C
   (Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Prod. No. P-5379. Adjust to pH 6.5 at 25°C with 1 M NaOH.)

B. 5.0 mM L-3,4-Dihydroxyphenylalanine (L-DOPA)
   (Prepare 10 ml in Reagent A using L-3,4-Dihydroxyphenylalanine, Prod. No. D-9628.)

C. 2.1 mM Ascorbic Acid Solution
   (Prepare 10 ml in Reagent A using L-Ascorbic Acid, Sodium Salt, Prod. No. A-7631. PREPARE FRESH.)

D. 0.065 mM Ethylenediaminetetraacetic Acid (EDTA)
   (Prepare 10 ml in Reagent A using Ethylenediaminetetraacetic Acid, Disodium, Dihydrate Salt, Stock No. ED55.)

E. Tyrosinase Enzyme Solution (PPO)
   (Immediately before use, prepare a solution containing 1000 – 3000 u/ml Tyrosinase in Reagent A.)
Enzymatic Assay of Tyrosinase 
Polyphenol Oxidase Activity 
(EC 1.14.18.1)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable quartz cuvettes:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A (Buffer)</td>
<td>2.60</td>
<td>2.80</td>
</tr>
<tr>
<td>Reagent B (L-DOPA)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent C (Ascorbic Acid)</td>
<td>0.10</td>
<td>------</td>
</tr>
<tr>
<td>Reagent D (EDTA)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 25°C. Monitor the $A_{265}$ nm until constant, using a suitably thermostatted spectrophotometer. Then add:

| Reagent E (TYR) | 0.02 | ------ |

Immediately mix by inversion and record the decrease in $A_{265}$ nm for approximately 5 minutes. Obtain the $\Delta A_{265}$ nm/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/mg enzyme} = \frac{\Delta A_{265} \text{nm/minute Test} - \Delta A_{265} \text{nm/minute Blank}}{(0.001) \text{ (mg enzyme/RM)}}$$

0.001 = The change in $A_{265}$ nm/min per unit of polyphenol oxidase in a 3.0 ml reaction mixture at pH 6.5 at 25°C.

RM = Reaction Mix

UNIT DEFINITION:

One unit is equal to a $\Delta A_{265}$ of 0.001 per min at pH 6.5 at 25°C in 3 ml reaction mix containing L-DOPA and L-ascorbic acid.

FINAL ASSAY CONCENTRATION:

In a 2.92 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 0.17 mM L-DOPA, 0.072 mM ascorbic acid, 0.0022 mM EDTA, and 20 - 60 units of tyrosinase.

Revised: 01/21/94
Enzymatic Assay of Tyrosinase
Polyphenol Oxidase Activity
(EC 1.14.18.1)

NOTES:

1. All products and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.

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SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of TYROSINASE
(EC 1.14.18.1)

PRINCIPLE:

\[ \text{L-Tyrosine} + \mathcal{O}_2 \rightarrow \text{L-DOPA} \]

\[ \text{L-DOPA} \rightarrow \text{L-DOPA-quinone} + \mathcal{H}_2\mathcal{O} \]

Abbreviation used:

\( \text{L-DOPA} = \text{L-3,4-Dihydroxyphenylalanine} \)

CONDITIONS: \( T = 25^\circ\text{C} \), pH = 6.5, \( A_{280\text{nm}} \), light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 50 mM Potassium Phosphate Buffer, pH 6.5 at 25°C
(Prepare 50 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 6.5 at 25°C with 1 M KOH.)

B. 1 mM L-Tyrosine Solution
(Prepare 100 ml in deionized water using L-Tyrosine, Free Base, Sigma Prod. No. T-3754.)

C. Tyrosinase Enzyme Solution
(Immediately before use, prepare a solution containing 500 - 1,000 units/ml of Tyrosinase in cold Reagent A.)

PROCEDURE:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container:

- Deionized Water: 9.00
- Reagent A (Buffer): 10.00
- Reagent B (Tyrosine): 10.00
Enzymatic Assay of TYROSINASE
(EC 1.14.18.1)

PROCEDURE: (continued)

Mix and adjust to pH 6.5 at 25°C with 1 M HCl or 1 M NaOH, if necessary. Immediately before use, oxygenate by bubbling 99.9% pure O₂ through the reaction cocktail for 3 to 5 minutes. Pipette (in milliliters) into suitable quartz cuvettes:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Cocktail 2.90</td>
<td>2.90</td>
<td></td>
</tr>
</tbody>
</table>

Equilibrate to 25°C. Monitor the A₂₈₀nm until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent A (Buffer) ------
Reagent C (Enzyme Solution) 0.10

Immediately mix by inversion and record the increase in A₂₈₀nm for approximately 10 minutes. Obtain the ΔA₂₈₀nm/minute using the maximum linear rate for both the test and blank.

CALCULATIONS:

\[
\text{Units/ml enzyme} = \frac{(\Delta A_{280\text{nm}}/\text{min Test} - \Delta A_{280\text{nm}}/\text{min Blank}) \times (df)}{(0.001) (0.1)}
\]

\( df = \) Dilution factor
0.001 = The change in A₂₈₀nm/minute per unit of Tyrosinase at pH 6.5 at 25°C in a 3 ml reaction mix
0.1 = Volume (in milliliters) of enzyme used

\[
\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}
\]

\[
\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}
\]

UNIT DEFINITION:

One unit will cause an increase in A₂₈₀nm of 0.001 per minute at pH 6.5 at 25°C in a 3 ml reaction mix containing L-tyrosine.

sPITRO01.001
Revised: 02/22/94
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FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 18 mM potassium phosphate, 0.3 mM L-tyrosine and 50 - 100 units tyrosinase.

REFERENCE:


NOTES:

1. Final volume of all cuvettes must equal 3 ml as stated in the Unit Definition.

2. This assay is based on the cited reference.

3. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

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